ELECTRICAL MOLECULAR FOCUSING FOR LASER INDUCED FLUORESCENCE BASED SINGLE DNA DETECTION

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ABSTRACT

This paper details an electrokinetic focusing technique that significantly enhances the detection efficiency for laser induced fluorescence (LIF) based molecular sensing by concentrating fluorescence labeled molecules in a tiny probe region. 3-D electrodes were fabricated to generate wider and denser electric fields toward the probe region in the microchannel. The generated electric field is able to focus flowing DNA molecules to widths as narrow as 3 µm in a 120 µm wide channel. A single molecule detection technique is developed and the enhancement of detection efficiency by using the focusing method is demonstrated.

INTRODUCTION

The detection and identification of molecules with high sensitivity is required in many fields, such as molecular biology, medical diagnostics, drug discovery, and forensic analysis. Recently, several micro biosensors have been designed to improve the S/N for specific oligonucleotide detection [1,2]. The advanced development of laser induced fluorescence (LIF) and confocal microscopy techniques have made possible the detection on the single molecule level. In the LIF based detection technique, molecules are detected in a limited focal region of a laser beam. Typically the probe region can be defined smaller than 100 fL. Background radiation caused by Rayleigh and Raman scattering of the laser beam by solvents is minimized due to the small number of solvent molecules present in such a small volume. Consequently, fluorescence bursts of single molecules that flow into the probe volume can be identified above the background.

On the other hand, because of its small probe volume, the LIF based detection has a potential problem with low detection efficiency, which is defined as the fraction of molecules in the analyzed sample that is actually detected. The efficiencies of on-column detection using conventional (50-100 µm i.d.) capillary columns are often less than 1%. Several techniques have been developed to enhance the detection efficiency, such as confining the sample stream to narrowed microchannels or ultramicrocapillaries [3], hydrodynamic stream focusing by creating a sheath flow [4], and molecular focusing by electric current [5]. Physical narrowing of microstructures results in two difficulties. First, the surface-to-volume ratio is too big in a tiny structure so that adsorption of the analyte at the channel surfaces may interfere with the measurements. Second, the limited passage may cause molecule clogged in the structure. The difficulties related to hydrodynamic and electric current focusing methods involve the alignment of the stream within the laser probe region. Any slight difference in conductivity or flow rate between the two focusing channels may cause the stream to drift off center [4,5].

In this paper, we developed a novel electrostatic molecular focusing technique by using 3-D electrodes. As shown in figure 1, the electrodes in the microchannel were designed to produce electric fields toward the probing region by applying the proper potential between the two side electrodes and the middle electrode. Before DNA molecules pass through the LIF probe region they are concentrated towards the middle electrode for detection by the applied electric field. Since the molecules are precisely focused to the downstream end of the middle electrode,

which is designed as the focal region of the LIF, more individually passing molecules can be sensed. As a result the detection efficiency is enhanced. This method overcomes the off-center problem encountered by the hydrodynamic and electric current focusing methods [4,5]. Additionally, unlike the other two focusing methods, this focusing technique does not necessarily rely on the continuous flow condition, which allows the usage of the static flow condition inside a closed receptacle, thereby broadening its applications.
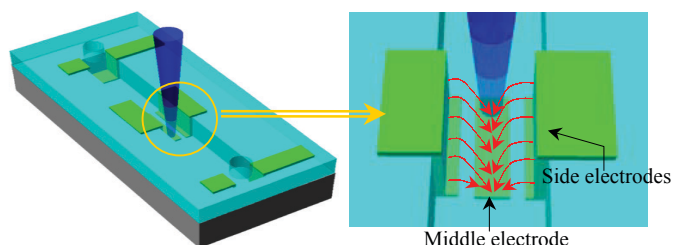
Figure 1. Conceptual schematics of 3-D electric molecular focusing. The middle electrode is applied with positive potential and both side electrodes are grounded. Negatively charged DNA molecules are concentrated to the middle electrode.
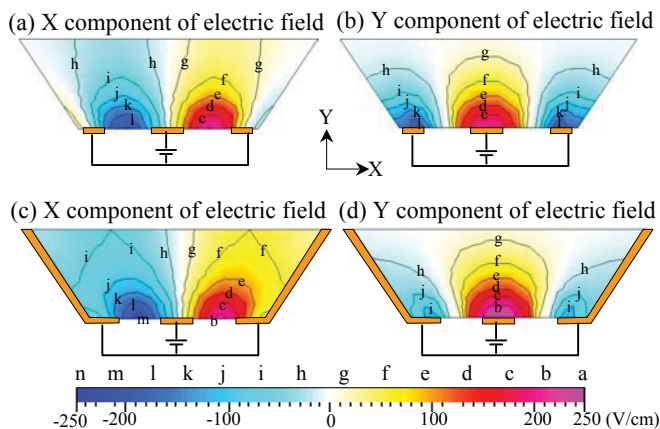
PRINCIPLES OF FOCUSING AND DESIGN OF ELECTRODES

The optimal design for focusing electrodes results in wider and denser electric fields that minimize the no-field or low-field regions (dead region) in the microchannel, therefore maximizing the number of molecules being detected. For a typical design of 2-D electrodes (Fig. 2(a), (b)) that are patterned on the bottom of a microchannel, the generated fields in the regions away from the planar electrodes are too weak to efficiently transport molecules to the probe region (Fig. 2(a)). Negatively charged DNA molecules near the side-walls and upper corners are not exposed to electric fields to move them to the middle region, where electric fields exist to push them downward to the detection area (Fig. 2(b)).

In order to stretch out the fields to fill the dead regions, we designed a 3-D electrode set (Fig. 2(c), 2(d)) that contains two side electrodes covering the side-walls and a middle electrode on the bottom of the microchannel. Electric fields are more densely and widely created from the middle to the side-electrodes, eliminating the dead regions of the design of the 2-D electrodes (Fig. 2(a)). As shown in figure 2(c), all DNA molecules (negatively charged) except those in the middle region experience electric fields and are electrophoretically moved inward to the middle region where downward pushing fields are produced (Fig. 2(d)). Another two electrodes were made in the inlet and outlet reservoirs to electrophoretically transport DNA molecules in the microchannel for flow-through detection (Fig. 1). Since molecules in upper regions experience lower electric fields (Fig. 2(a), 2(c)), it takes a longer time for them to reach the probe region that is aligned on the downstream end of the middle electrode. Some molecules may have already passed through the probe region and are not able to be detected. Therefore, to have higher detection efficiency the focusing electrodes need to be extended forward

enough to allow sufficient focusing time for the molecules to reach the probe region and be detected. The required focusing length needs to be designed according the magnitude of the applied focusing fields, the ratio between strengths of moving electric fields and focusing electric fields, and the geometry of the electrodes.

Another feature of this focusing design is that the distance between electrodes is small, ranging from only 20 to 100 μm. Therefore, even when the applied voltage is as low as 1 V, the generated electric fields can be greater than 100 V/cm. Since low voltage is applied, the focusing can be implemented without generating bubbles due to electrolysis.

(a) X component of electric field   (b) Y component of electric field

n   m   l   k   j   i   h   g   f   e   d   c   b   a

-250   -200   -100   0   100   200   250 (V/cm)

Figure 2. Simulation of electric fields generated from 2-D electrodes((a) and(b)) , and from 3-D electrodes((c) and (d)). The applied voltage set for simulation is 1V.
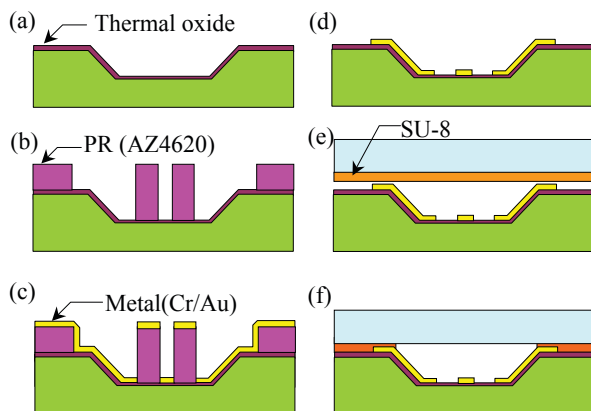
Figure 3. Process flow of a molecular focusing chip with 3-D electrodes.

FABRICATION

Microchannels were fabricated on a silicon substrate by KOH etching to have smooth and tapered side-walls for better metallic coverage (Fig. 3). The upper channel width is 120 μm and the depth is 30 μm. After thermal silicon oxide was grown to a thickness of 5000Å for electric isolation, a 200 Å /2000Å Cr/Au layer was deposited by e-beam evaporation. To pattern 3-D electrodes on the top and bottom of the channel, 10 μm thick PR AZ4620 was over-exposed and developed for lift-off [1]. The width of the middle electrode was fabricated to be 20 μm with 20

μm space from the side electrodes. Pre-drilled Pyrex glass plates were spin-coated with SU-8 and cured at 85 ^{0}C for 3 minutes before bonding. The channel chips were then bonded with the glass plates, and the bonded chips were further cured at 95 ^{0}C for 10 minutes to remove excess solvent to prepare for UV exposure and patterning for clearing purposes.
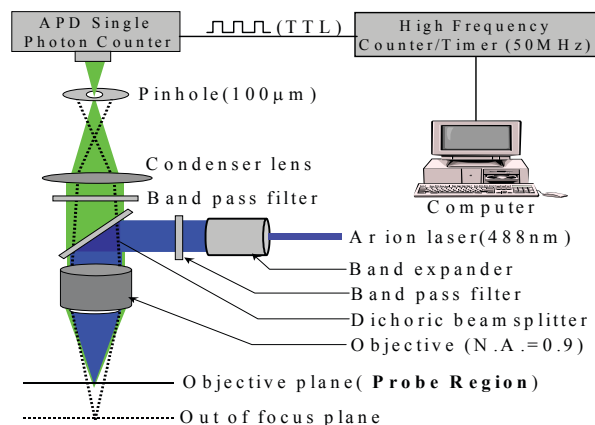
Figure 4. Schematic diagram of the experimental (Confocal Laser Induced Fluorescence System) setup.

INSTRUMENTATION

A Laser Induced Fluorescence (LIF) system (Fig. 4) is used for fluorescence excitation. A light beam (0.15 mW) from an air-cooled Ar ion laser (Melles Griot, 35LAL415-220) passes into a beam expander (Melles Griot, 09LBZ010) and a band pass filter (Omega, XF1073). It then reflects from a dichroic beam splitter (Omega, XF2037) to a 50 x 0.90 N.A. oil immersion objective (Olympus), which focuses the beam to a 3 μm spot within the channel. Fluorescence is collected by the same objective, passes through a dichroic beam splitter, filtered by a bandpass filter (Omega, XF3003), focused by a focusing lens (Newport, PAC052), focused through a 100 μm pinhole, and finally collected by an avalanche photodiode (Perkin Elmer, SPCM-AQR). Amplified TTL level pulses were counted by a PC plug-in board (PC100D, Advanced Research Instruments) and stored. A program written in C was created to perform data acquisition and to set bin width (integration time per data point) of counting. Bin widths could be set to power of 2, eg. 64, 128, or 256 μs per bin.

SAMPLE PREPARATION

λ DNA digest (6 different DNA fragments: 3530, 4878, 5643, 5806, 7421, 21226 bp), λ DNA (48 Kbp), and T2 (164 Kbp) were purchased from Sigma and were used to characterize the molecular focusing. The DNA sample was diluted in 1X Tris-borate buffer (TBE) to the desired concentrations and was stained with YOYO-1 iodide (Molecular Probes, Y3601). The YOYO-1 solution was diluted in DI water to the desired concentration before mixing with the DNA. The base pair to dye molecule ratio was kept at 5:1 for the DNA solution to have better single to noise ratio. The resulting solution was incubated for 30-60 min at room temperature in the dark and diluted in TBE to give a final concentration of ~ 1 × 10 $^{-14}$ M for the single molecule detection experiments.

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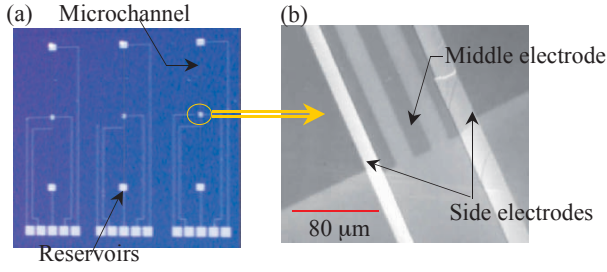
Figure 5. (a) A picture of the molecular focusing chip with three sensors, (b) A SEM picture of the 3-D focusing electrodes.
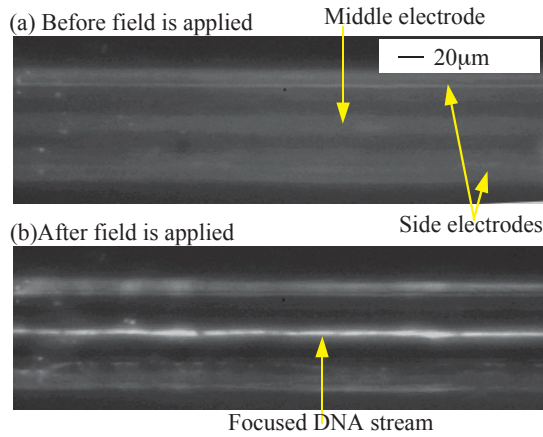

Figure 6 CCD images of focusing for smaller DNA, λ DNA digest in a static flow condition.

EXPERIMENTAL RESULTS

The picture of a 3-channel array is shown in figure 5(a), and the SEM picture of the focusing electrodes is presented in figure 5(b). To create focusing fields, a 10 kHz pulsed voltage (1.4 Vpp) with 0.7 V offset was applied to the middle electrode with the two side electrodes grounded. Figure 6(a) and 6(b) demonstrate the design's effectiveness in focusing DNA molecules in a static flow condition. In order to have enough fluorescence intensity for taking images, a higher concentration of λ DNA digest solution (2×10^{-11}M) was introduced in the channel. Before applying the focusing fields, the DNA molecules were uniformly distributed in the microchannel (Fig. 6(a)). After the potential was applied, the DNA molecules were focused to a narrow strip in the middle of the middle electrode (Fig. 6(b)). As shown in the figure, DNA molecules were confined in a strip as narrow as ~2 μm. The serial pictures in Figure 7 demonstrate a process to focus and defocus DNA molecules in a continuous flow condition. Larger DNA (T2) with lower concentration 10^{-12}M were used in order to have imagines of individual DNA. To transport DNA from upstream (left) to down stream (right), a 5V potential was applied between the electrodes in the reservoirs. Before applying the focusing fields (Fig.7 (a)), DNA moved uniformly from left to right. Since the microscope was focused in proximity to the bottom of the channel, only a few DNA molecules could be clearly observed in this picture. After the focusing fields were applied (Fig. 7(b), 7(c), and 7(d)), DNA in the top and side regions of the channel were

gradually driven to the bottom of the channel while flowing downstream. That more DNA observed in the focal planes shows the evidence of DNA moving downward to the middle electrode. In the continuous flow condition, the molecules downstream have been exposed to the focusing fields longer than the molecules upstream, therefore they were more narrowly confined in the middle of the middle electrode. As a result, the focusing efficiency (detection efficiency for LIF based detection) in downstream is larger than upstream. Fig 7(d) shows that the DNA were focused to widths of only 3μm. This implies the detection efficiency can be greatly enhanced if the LIF detection is performed on the downstream end of the focusing electrode. On the other hand, to demonstrate the defocusing effects, a positive pulsed voltage (V_{pp} =1.4 V, V_{offset}= 0.7V) was applied to side electrodes while keeping the middle electrode grounded. The DNA were immediately pulled outward and upward and were defocused as shown in figure 7(e).
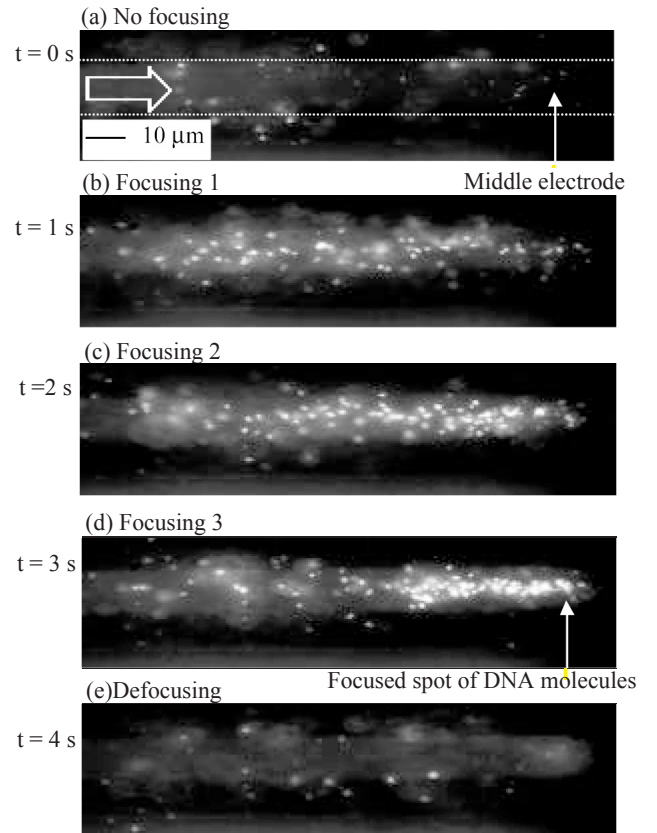
Figure 7 CCD images of focusing and defocusing larger T2 DNA in a continuous flow condition.

To characterize the improvement of detection efficiency for single molecule detection by using the same focusing chips, a confocal LIF system (Fig. 4) with an APD based single-photon counter was used. The e^{-2} probe volume in the case was approximated to be a cylinder 3.0 μm in diameter and 4 μm in height that gives a volume of 28 fL. A more diluted λ DNA solution, 10^{-14} M stained with YOYO-1 was flowed through the microchannel. When DNA solutions were introduced, discrete fluorescence bursts were seen due to the passage of individual DNA molecules through the focused laser beam. The probability of more than one DNA molecule simultaneously occupying the probe volume can be calculated using the concentration (10^{-14} M) and
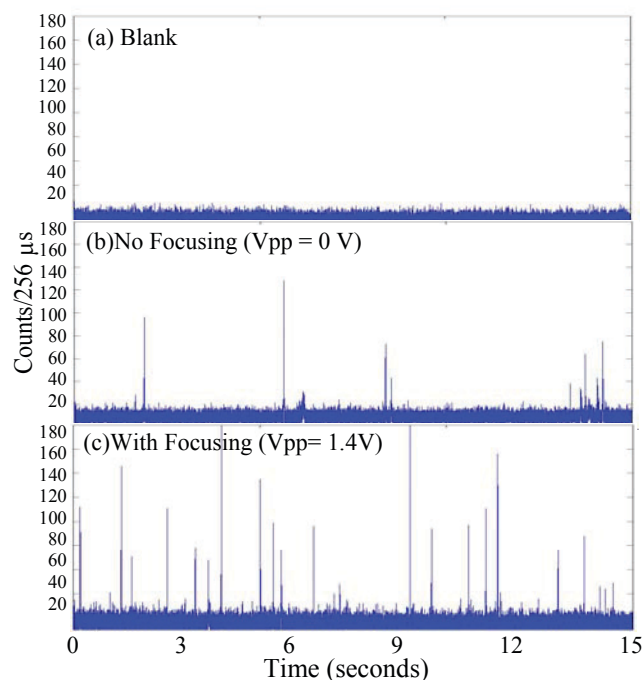
Figure 8. Detection of single λ DNA bursts. Detection with Vpp =1.4V applied to the probe region, The DNA concentration is 20 fM.

Figure 9. Autocorrelation functions calculated from the λ DNA solution. (a) Unnormalized autocorrelation functions. (b) Normalized autocorrelation functions.

Figure 8 presents a fifteen-second span of single molecule bursts events while DNA passing through the probe volume. The time bin for each counting was set as 256 μs. When the blank solution was flowed through the channel, no large fluorescence bursts were observed (Fig. 8(a)). When DNA solution was introduced, discrete burst events began to be seen. The single molecule bursts were rarely observed without proper molecular focusing (Fig. 8(b)). After the electric field was applied to concentrate the DNA molecules in the probe region, the frequency of the single molecule bursts was greatly increased (Fig. 8(c)). The autocorrelation functions were calculated to demonstrate the presence of non-Poissonian bursts due to single molecules (Fig. 9). The formula used to calculate the autocorrelation function was $G(\tau)=(1/N)\Sigma n(t)n(t+\tau)$, where G is the autocorrelation, N is the size of the data set, n is the value at time t, and τ is the offset. The unnormalized autocorrelations in figure 9(a) show that the magnitude of the autocorrelations increases when the electric focusing fields were applied, and the normalized autocorrelations in figure 9(b) show that the shape and width of the autocorrelation functions change very little with the fields, indicating uniform transit times which is expected for single molecule detection. Based on the experimental data, the average improvement of detection efficiency can be more than 5-fold. Further improvements can be achieved by optimizing experimental factors such as the geometry of channel and electrodes, and electric conditions.

CONCLUSION

A high efficiency molecular focusing method for LIF based single molecule detection was achieved by electrostatically manipulating DNA in a microchannel. A 3-D electrode set was fabricated in the channel to create an electric field that is able to focus DNA molecules in a 3μm wide strip that is comparable with the probe size of the LIF, thereby enhancing the detection efficiency. Compared with other molecular focusing techniques such as hydrodynamic focusing and electric current focusing, our method
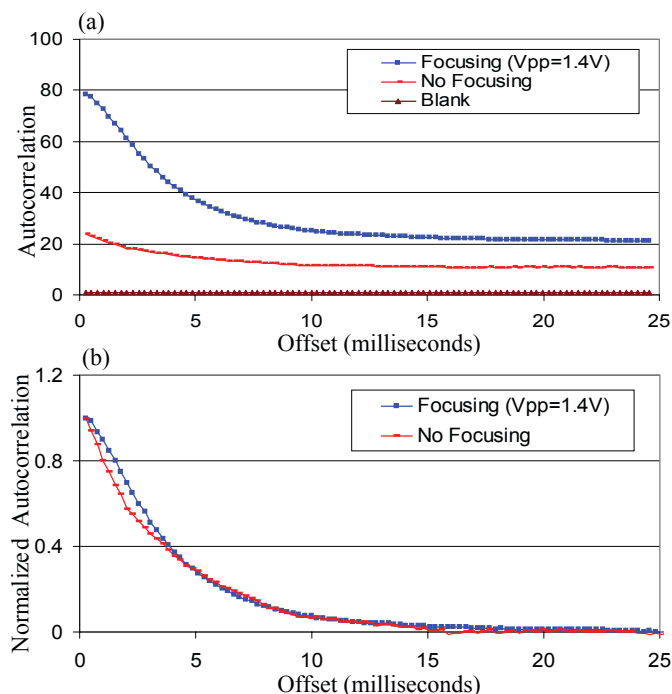
not only has superior detection efficiency but it also overcomes the off-center problems found in focused sample streams. It can also be applied in a static flow condition that broadens its applications.

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